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Biological Nitrogen Fixation, 1924–1974

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Crop rotation was practiced in ancient times by alternating the use of leguminous and nonleguminous plants. The basis for the benefit derived from this practice was not understood, but it was observed empirically that crops were improved and the condition of the soil was maintained better with rotation than by continual cropping with nonleguminous plants. Rotation was recognized as good husbandry, but it remained for Boussingault to offer an explanation of the benefit in the 1830's. He established one of the earliest agricultural experiment stations and demonstrated in the greenhouse that clover and peas increased their nitrogen content when grown on sand that was virtually free of fixed nitrogen. His field experiments likewise supported the view that leguminous plants could use N_2 from the atmosphere.

The observations of Boussingault touched off heated controversy and Liebig suggested that the observed increases in nitrogen in the leguminous crops arose merely because the large leaved leguminous plants were capable of extracting more ammonia from the atmosphere than were the smaller leaved control plants. Because Liebig could speak with authority in the area of organic chemistry, he apparently felt that this carried with it the privilege of speaking in the area of agricultural chemistry without the need to present experimental evidence in support of his postulates. It is hardly surprising that a French commission, assigned to the task of checking to see whether Liebig or Boussingault were correct, came to the conclusion that their fellow countryman in fact had demonstrated an increase in nitrogen in the leguminous plants.

N_2-FIXING ORGANISMS

Do Plants Fix N_2? The experiments performed by the French commission of scientists did not convince everyone, and hence, Lawes *et al.* (29) felt compelled to put the hypothesis of biological N_2 fixation to the crucial test. They devised an experiment which was a model of thoroughness and they analyzed not only the plants, but the soil and even the pots in which the plants were grown. In preparation for the experiment, they heated the sandy soil to a high temperature to destroy organic material, and in the process killed the root nodule bacteria which are responsible for fixation of N_2 in association with leguminous plants. Thus, excessive care in experimentation destroyed the agents of fixation and led them to the conclusion that biological fixation of N_2 did not occur. Their experiments were performed so carefully that they appeared definitive in answering the question posed. However, there were a few skeptics, such as Atwater (1) in the United States, who continued to report evidence of biological fixation of N_2.

The truly definitive experiments were performed by Hellriegel and Wilfarth in Germany; they reported their results at a meeting in Berlin in 1886 and published a comprehensive paper in 1888 (23). They were interested in nitrate nutrition of plants and in their experiments included control plants to which no nitrate was added. The plants were grown on a sand substrate to minimize the amount of nitrogen supplied by the soil, and the growth response of peas was irregular. Some flourished on the nitrogen-free medium, whereas others showed minimal growth. Hellriegel and Wilfarth observed that the healthy green plants in every instance carried nodules on their root system. These leguminous nodules had been described on plants centuries earlier, but no one had recognized the significant fact that this was the seat of biological N_2 fixation.

Hellriegel and Wilfarth repeated their experiments but incorporated treatments in which soil was used to inoculate the seeds. As in the early experiments, the uninoculated controls showed an erratic response, dependent upon whether they were subject to chance inoculation. Plants inoculated with soil showed uniformly good growth, even on the nitrogen-deficient soil, and they were uniformly nodulated. Thus, Hellriegel and Wilfarth established the reality of biological N_2 fixation and showed that N_2 fixation is associated with the nodules of leguminous plants; the implication was clear that the nodules were induced by microorganisms.

Exploitation of N_2 Fixation. It was apparent from the experiments of Hellriegel and Wilfarth that biological fixation of N_2 had tremendous implications for agricultural practice. There were no adequate methods for the chemical fixation of N_2 and agriculturalists were dependent upon the use of manures, guano, and plant residues to support the nitrogen needs of their crop plants. The clear-cut demonstration that one of the macronutrient elements for plants could be extracted from the inexhaustible reservoir of the air prompted agronomic and bacteriological investigations to determine the best methods to exploit the finding.

Although crops could be inoculated with soil from fields which had previously grown the same crop, it soon was established that controlled culture of the proper microorganisms furnished a more reliable source of organisms to add to the soil or to the seed at the time of planting. The production of commercial inoculants had a rather spotty history in the early days. Although some reputable groups supplied highly active cultures, there were numerous manufacturers with low standards who had little concern for the effectiveness of the bacteria or the viability of the cultures sold. Eventually, the production of inoculum came under government control or testing in many countries, and for many years it has been possible to buy highly effective inoculant in a convenient form for application to seed.

It is hardly surprising that early studies on N_2 fixation emphasized the application of the N_2-fixing system in practical agriculture. It was soon recognized that there was a marked difference in effectiveness of N_2 fixation among bacterial strains capable of producing root nodules. It also was apparent that some rhizobia would infect a specific leguminous plant, whereas others would not. The concept of cross-inoculation groups grew from this practical observation that only specific

organisms could invade the roots of specific groups of plants. Inoculant was sold on the basis of its ability to infect a particular group of leguminous plants. It remains a common practice to mix strains of the bacteria to insure the presence of organisms that are effective on a variety of species and strains of legumes.

There was considerable curiosity about the properties of the root nodule bacteria, and hence their physiological properties were studied in detail. One problem that was particularly intriguing, but difficult to resolve, was whether or not the root nodule bacteria fixed N_2 when grown by themselves. If you examine the history of the subject as summarized in the monograph by Fred *et al.* (19), you will find almost an equal split between those who found fixation of N_2 by free-living rhizobia and those who found no fixation. Characteristically, the experiments were run over long periods and the observed fixation was small. In some experiments yielding positive results after prolonged incubation, ammonia may well have been accumulated from the atmosphere by the organisms. In other instances, analytical errors may have played a part. After 1930, the number of positive reports dwindled, and when ^{15}N became available as a tracer, it was possible to test the hypothesis with a much more sensitive technique. No reliable tests with ^{15}N ever demonstrated any fixation by free-living rhizobia.

Free-living N_2-fixing Organisms. The clear establishment that biological N_2 fixation could occur prompted a search for organisms which could achieve fixation apart from any association with a host plant. Winogradsky (50) showed that certain clostridia were capable of fixing N_2 and Beijerinck (3) showed that the azotobacter were representative of aerobic organisms which were capable of N_2 fixation. Although there were indications that the blue-green algae could fix N_2, these organisms commonly were contaminated with bacteria, and hence some question remained about their ability to fix N_2. In 1928, Drewes (16) obtained cultures of *Nostoc* and *Anabaena* species free from bacterial contamination, and showed that they were able to fix N_2. Allegations that other groups of organisms could fix N_2 were not supported by convincing evidence, although there is a long history of claims for fixation by uninoculated legume seed, willow cuttings, fungi, and organisms as complex as whales. In recent years, it has been possible to test these claims with more definitive methods, and notable additions have been made to the list of N_2-fixing organisms. It is particularly interesting that the heavily studied photosynthetic bacteria all proved to be capable of N_2 fixation. This was first recorded by Kamen and Gest for *Rhodospirillum rubrum* (25). It also is interesting that certain facultative and microaerophilic organisms can fix N_2 under anaerobic conditions. A number of these organisms, after initial misidentification, turned out to be members of the genus *Klebsiella. Bacillus polymyxa, Desulfovibrio sp.,* and certain methane bacteria also fix N_2 anaerobically.

Much of the early work on N_2 fixation was reviewed thoroughly in the monograph by Fred *et al.* (19). Anyone who feels that our knowledge of biological N_2 fixation has all been acquired in the last decade would be disabused of the idea by reading this 1932 monograph. Not only were the cultural, morphological, and physiological aspects of the organisms discussed, but considerable attention was paid to the cross-inoculation groups, the invasion of the root by the nodule bacteria, the formation and morphology of the nodule, practical aspects of inoculation, and quantities of nitrogen fixed in the laboratory and field. There also was a good deal of attention given to the nonleguminous plants capable of fixing N_2.

BIOCHEMISTRY OF N_2 FIXATION

Initiation of Biochemical Studies. A limited number of experiments which could be classified as biochemical in their approach were performed in the period between Hellriegel and Wilfarth's experiments in 1886 and the late 1920's. Fred *et al.* (19) had a chapter entitled, "Cultural and Biochemical Characteristics of the Root Nodule Bacteria." The discussion was centered around the nitrogen nutrition of the organisms, their capacity for liquifaction of gelatin, and the changes they produced in a milk medium. There were data for the fermentation of a variety of sugars, for the enzymes involved in reduction of indicator dyes, and for the bacterial production of hydrogen sulfide and ammonia. Rough analyses had been made on the gross composition of the rhizobia and *Azotobacter chroococcum.*

Serious biochemical work on N_2 fixation appears to have started in the late 1920's. In 1928, Meyerhof and Burk (33) published work describing the influence of the partial pressure of N_2 on the fixation process. Burk had received his degree at the University of California at Berkeley and had gone to Europe to study photosynthesis and N_2 fixation. His interest in the physical-chemical properties of the N_2-fixing enzyme system prompted his investigations in Europe and he continued these after returning to the Fixed Nitrogen Laboratory in Washington, D. C. Here, he and his colleagues pursued biochemical studies of the process through the 1930's. Every beginning student in biochemistry is acquainted with the Lineweaver-Burk double reciprocal plot to express the relationship of substrate concentration and reaction velocity.

One of the more interesting interludes in Burk's investigations was his attempt to repeat the experiments of Bach *et al.* (2) that led to Bach's claim that cell-free N_2 fixation could be obtained with extracts from *Azotobacter chroococcum.* The group at the Fixed Nitrogen Laboratory had tried repeatedly and unsuccessfully to obtain cell-free fixation as described by Bach *et al* (2). Burk attended a conference in Russia, had an opportunity to talk with Professor Bach, and was invited to spend some time in Bach's laboratory. Burk received permission and support to stay in Moscow for a couple of months to investigate cell-free N_2 fixation. There had been some changes in the personnel in Bach's laboratory and the new group was entirely unsuccessful in demonstrating fixation of N_2 by cell-free preparations. Burk was inclined to think that the earlier group may have had some success, but our present knowledge about the conditions under which cell-free fixation is achieved make this seem unlikely. Everyone was very interested in the outcome of the experiments, and Burk was quizzed extensively at each of the institutions where he stopped in Europe on his way back to the United States. Bach's group subsequently had no success in repeating the experiments and they published a single communication on the cell-free fixation. Undoubtedly, many people were unsuccessful in repeating Bach's work, but these negative results remained unpublished. Roberg (40) did publish the results of his unsuccessful attempts to obtain cell-free fixation.

I believe that Burk's final contribution to the field of N_2 fixation was a review article that he and I wrote for the 1941 Annual Review of Biochemistry (9). This summarized our viewpoints on the subject, and in a few matters we noted that we were at odds. Burk and I ran a few joint experiments, and I was pleased to convince him that H_2 was a competitive and specific inhibitor for N_2 fixation in *Azotobacter vinelandii,* a point which he had long rejected.

The physical-chemical studies that Burk had initiated were expanded by Perry W. Wilson and his group and extended to the symbiotic N_2-fixing system. Wilson was a student at Rose

Polytech in Terre Haute, Ind. and a lab helper at the Commercial Solvents plant at the time he was "discovered" by Fred and Baldwin when they were consulting with Commercial Solvents regarding phage problems in the *Clostridium acetobutylicum* fermentation. Wilson was encouraged to come to the University of Wisconsin to complete his B.S. degree and to continue graduate studies in bacteriology and biochemistry. Fred, who had initiated his work on N_2 fixation at the University of Wisconsin in 1913, directed Wilson's research. After receiving his Ph.D. degree in 1932, Wilson set up shop in the Department of Bacteriology. These were lean times, and a Frasch Foundation grant of a few thousand dollars per year put Wilson's lab in a state of relative opulence. I worked during the summer of 1935 in Wilson's lab and joined the group as a graduate student in 1936. To me, history of N_2 fixation prior to 1935 is something derived from books and journals, but after 1935 it represents a personal experience colored, tempered, and prejudiced by my acquaintance with contributors in the field. My experiences cover almost 40 years rather than the 50-year span of the American Society of Plant Physiologists; the golden anniversary offers an excuse to concentrate on the past half century rather than the whole historical span of N_2 fixation.

Burk had concentrated on *Azotobacter chroococcum*. The symbiotic system, generally red clover plus *Rhizobium trifolii*, was difficult to use experimentally because the plants had to be grown under bacteriologically controlled conditions in closed bottles. The bottles generally used were 9-liter Pyrex serum bottles closed with a rubber stopper holding inlet and outlet tubes. A sand substrate with nitrogen-free nutrient was supplied. The bottles could be evacuated and the atmosphere above the plants replaced with the desired mixture of gases. An internal colorimetric indicator showed when CO_2 was needed. On a sunny day, a substantial portion of a graduate student's time was spent filling the bottles with CO_2. Although the system was cumbersome and demanding, it sufficed to establish the response of the symbiotic N_2 fixation system to the partial pressure of N_2 and O_2. It also revealed that H_2 was a specific and competitive inhibitor of N_2 fixation.

This remarkable observation that H_2 is an inhibitor met with skepticism, and it was necessary to show in rigorous fashion that the inhibition came from H_2 itself rather than from some impurity in the gas. Tank H_2 was scrubbed through a series of absorbants and passed through a furnace to remove any inhibitors. There were those in the laboratory who were leery of passing H_2 through a furnace, despite assurances that nothing would happen as long as O_2 was excluded. H_2 was generated from a variety of sources, the rationale being that different sources would be unlikely to carry the same inhibitor. I generated H_2 from Zn and HCl, from NaOH and Al, and electrolytically. Umbreit tested all of these, and in every case the inhibition by H_2 was the same (49). H_2 remains the most specific of the inhibitors for biological N_2 fixation.

Wilson mounted a remarkably broad attack on the problems of N_2 fixation. Louise Wipf investigated the process of invasion and nodule formation and concluded that only the spontaneously polyploid cells were invaded by the rhizobia. Umbreit investigated the changes in nitrogenous composition of leguminous plants occurring under different treatments. Phil West established that biotin was a specific requirement for the growth of the rhizobia. Joe Burton and Orville Wyss investigated the excretion of fixed nitrogen from leguminous plants, and Wyss, Joe Wilson, and Sylvan Lee demonstrated H_2 inhibition in the free-living azotobacter. W. Thorne, Richard Tam, and I studied the respiration of the rhizobia as recovered from nodules and as grown on laboratory media. In collaboration with Fred, Wilson (47) had investigated the role of the carbohydrate-nitrogen relationship in N_2 fixation, a subject which occupies all of Chapter 6 in Wilson's 1940 monograph on N_2 fixation. This subject had been a favorite with plant physiologists, and much earlier had been discussed in detail by Schulze and by Prianischnikow. It is interesting that within the last year or so, studies of the carbohydrate-nitrogen relationship have been revived, and once more it has been discovered that an increase in CO_2 concentration will enhance N_2 fixation.

Virtanen (45) directed another laboratory group which vigorously and consistently investigated the biochemical problems of N_2 fixation. His work centered around the observation that leguminous plants inoculated with the rhizobia, but free from other contaminating bacteria, excreted fixed nitrogen compounds into the medium. His analysis suggested that the prime product of excretion was aspartic acid and its decarboxylation product β-alanine. These limited data served as the cornerstone for Virtanen's vigorous championing of hydroxylamine as the key intermediate in biological N_2 fixation. The key intermediate was defined as the product which marked the end of N_2 fixation and the initiation of assimilation of nitrogen into organic combination. Other groups had little success in demonstrating excretion of nitrogen. In 1936, Wilson spent time in the Department of Biochemistry at Cambridge, England, mastering the techniques of studying respiratory enzymes. He also did research on excretion of nitrogen in Virtanen's laboratory in Helsinki. Excretion was positive, but very limited. As Wilson had had no success in demonstrating excretion of nitrogen when he was in Madison, Wisconsin, he returned with the culture and seed used by Virtanen and also imported a large box of Finnish sand. Experiments were set up exactly as had been done in Helsinki and again the results were negative. The graduate students then were asked to set up experiments the first of each month ("marathon experiments") to see whether seasonal changes were responsible for excretion; results generally were negative. Dr. Roberts of the Department of Horticulture at the University of Wisconsin was studying effects of temperature and day length and observed excretion to a nonleguminous indicator plant a number of times under conditions of low temperature, low light intensity, and long day. These are the conditions which prevail in Helsinki, and apparently they help to induce excretion.

Virtanen concluded that because aspartic acid was excreted from the plant it had a special significance as an intermediate in N_2 fixation. He reasoned that hydroxylamine reacts vigorously with oxaloacetic acid, and hence these compounds must combine to form the corresponding oxime which in turn is reduced to aspartic acid. The excretion of aspartic acid provided only questionable evidence for its role as an intermediate in N_2 fixation. Virtanen pointed out that oxaloacetic acid is formed in plants, but this can be said equally well for a number of other ketoacids. The claim that the addition of oxaloacetic acid to excised leguminous root nodules enhanced their N_2 fixation was never verified adequately; variations between replicate samples of nodules were large relative to claimed increases in fixed nitrogen. Virtanen and Laine (46) reported isolation of the copper salt of oximinosuccinic acid; this was the most specific evidence in support of his hypothesis, but to this day it has not been verified in any other laboratory. With ^{15}N as a tracer, we were able to demonstrate that a variety of other nitrogenous compounds, in addition to aspartic acid, were excreted from leguminous plants under controlled conditions. We never felt it necessary to publish these results, however, as after World War II, Virtanen's own data indicated that compounds, other than aspartic acid, were present among the excretion products.

Although Virtanen maintained a vigorous program in biological N_2 fixation for many years and contributed many interesting and helpful observations, the central theme of his work, that hydroxylamine was the key intermediate in N_2 fixation, proved to be incorrect.

Work at the Fixed Nitrogen Laboratory, work in Wilson's laboratory at Wisconsin, and work in Virtanen's laboratory in Helsinki, constituted the backbone of investigations in the area of the biochemistry of N_2 fixation for many years. To be sure, sallies were made into the area by a number of groups, but they did not sustain long term programs of biochemical investigation.

It can hardly be said that in 1940 study of the biochemistry of N_2 fixation was popular. In 1940, Wilson published his monograph (47) and dedicated it to his mentor and major professor, Fred. This constitutes an excellent summary of the state of the subject in 1940, and if you read this volume, I am sure you will be impressed with the amount of information then available on the topic.

Application of ^{15}N as a Tracer. In the period 1940–1960, the use of ^{15}N as a tracer had substantial impact on studies of the biochemistry of N_2 fixation. When H. C. Urey concentrated ^{15}N and when Schoenheimer, Rittenberg, and colleagues applied ^{15}N in studies of animal metabolism, it became apparent that it could be of real use in the study of biological N_2 fixation. Upon receiving my Ph.D. at the University of Wisconsin in 1940, I obtained a National Research Council Postdoctoral Fellowship and spent the year at Columbia University studying in the laboratories of H. C. Urey, S. F. Trelease, and R. Schoenheimer. Urey was most gracious in supplying the concentrated ^{15}N, and he immediately grasped the significance of utilizing the isotope in studies of N_2 fixation. C. Miller and I demonstrated that there was no measurable exchange reaction catalyzed by *Azotobacter vinelandii* and concluded that ^{15}N could be used as a valid tracer to detect net fixation of N_2.

The isotope was used to show that nonleguminous plants exposed to $^{15}N_2$ for an extended period did not fix N_2, whereas inoculated leguminous plants fixed N_2 actively. This helped to inter the recurring claims for fixation by nonlegumes under aseptic conditions. To get some insight into the products of N_2 fixation, I exposed growing cultures of *Azotobacter vinelandii* to $^{15}N_2$ for 90 min, inactivated the organisms, isolated their amino acids, and determined the $^{15}N_2$ concentration in the amino acids. This was done in Dr. Schoenheimer's laboratory, and not only did he give me a hand, but I was also aided by Sarah Ratner, David Rittenberg, and Konrad Bloch. Recovery of amino acids from a hydrolysate at that time involved formation of crystalline derivatives of each amino acid to be analyzed. It is difficult in this era of ion exchange columns to recall the effort that went into such investigations. In these studies, with the azotobacter exposed to $^{15}N_2$, the highest concentration of ^{15}N appeared in glutamic acid. Acceptance of Virtanen's hydroxylamine hypothesis required that the highest concentration of ^{15}N appear in aspartic acid if it were the first product of nitrogen assimilation. The high concentration of ^{15}N in glutamic acid was compatible with the formation of ammonia from N_2 and its assimilation by reductive amination of α-ketoglutarate.

By supplying a variety of unlabeled fixed nitrogen compounds to *Azotobacter vinelandii* simultaneously with $^{15}N_2$, it was possible to distinguish which sources were preferred by the organism. It was apparent that there was something special about ammonia, because it completely suppressed fixation and was used to the virtual exclusion of other compounds. A number of lines of evidence pointed strongly to ammonia as the key intermediate; although not overwhelming, the data certainly were much stronger than any marshalled in support of the hydroxylamine hypothesis. More by frequent repetition of claims than by inherent merit, the hydroxylamine hypothesis was receiving acceptance and was being incorporated into textbooks; so in 1946, Wilson and I felt compelled to assert our support of ammonia (12). In 1947, we reviewed all the evidence available at the time in support of the ammonia hypothesis (48). A paper by Zelitch *et al.* in 1951 (51) added the data that seemed to clinch the ammonia hypothesis of N_2 fixation. When formation of the ketoacid acceptor for ammonia was limiting, *Clostridium pasteurianum* excreted ammonia into the medium, and when $^{15}N_2$ was supplied under such conditions, the ammonia which was excreted had an unusually high concentration of ^{15}N. This constituted direct evidence for ammonia as an intermediate in the process and reinforced the other lines of evidence which, in aggregate, supplied convincing support for the ammonia hypothesis. Since that time, the ammonia hypothesis has been accepted generally, although someone occasionally has raised a question to demonstrate his openmindedness on the issue.

The use of ^{15}N as a tracer was also helpful in demonstrating a number of new agents capable of fixing N_2. In 1949, Kamen and Gest (25) demonstrated that *Rhodospirillum rubrum* fixes N_2. They came to our laboratory to check their observations with ^{15}N as a tracer, and obtained clear-cut, positive results for fixation. The experiments were easy to verify, and Lindstrom *et al.* (30) extended observations of N_2 fixation to the other types of photosynthetic bacteria. It is particularly interesting that an organism as extensively studied by bacterial physiologists as *Rhodospirillum rubrum* had not been recognized as a N_2 fixer until 1949. Later, it was possible to show that *Bacillus polymyxa* and *Klebsiella pneumoniae* could fix N_2 under anaerobic conditions. The use of ^{15}N as tracer also expanded the number of blue-green algae which were recognized as N_2 fixers.

Cell-free N_2 Fixation. Achieving cell-free N_2 fixation proved to be more difficult than anticipated. As indicated, the early work by Bach and co-workers was not confirmed in any other laboratory and could not be repeated by his own group. Because the ^{15}N tracer method was at least 100 times more sensitive than the total nitrogen analyses used by Bach and others, it seemed that cell-free fixation should be relatively easy to demonstrate with the tracer. In the early 1940's, we produced N_2-fixing bacteria in quantity, learned methods for disrupting them, and recovered a variety of cell-free enzymes other than nitrogenase. Our criterion for positive fixation of $^{15}N_2$ was an incorporation of 0.015 atom % ^{15}N excess above the normal abundance into the preparation. This was a conservative criterion, because our mass spectrometer easily distinguished 0.003 atom % ^{15}N excess. Based on the standard of 0.015 atom % ^{15}N excess, we obtained many positive preparations, primarily from extracts of *Azotobacter vinelandii* and *Clostridium pasteurianum*. Both aerobic and anaerobic conditions were tried and various substrates were supplied. Results with *Azotobacter vinelandii* (27% positive) were summarized by Magee and Burris (31), and Hoch and Westlake (24) reported positive fixation by *Clostridium pasteurianum*. These and other early data on cell-free fixation were summarized in 1966 (11). Although there were numerous clear-cut cases of positive N_2 fixation, it must be said in all honesty that these were of no particular use for the study of the mechanism of N_2 fixation, because N_2 fixation was not vigorous and it was inconsistent from experiment to experiment. In late 1959, we were obtaining rather consistent fixation with extracts from blue-green algae, when Carnahan *et al.* (13) developed a method that yielded extracts of *Clostridium pasteurianum* that supported

vigorous and consistent fixation of N_2. This marked a turning point in the study of N_2 fixation. We quickly reproduced the results and started to utilize the preparations from *Clostridium pasteurianum*, as they were superior to the extracts from blue-green algae. There were three tricks in acquiring vigorous fixation: first, all operations were anaerobic, which was rather obvious with an organism such as *Clostridium pasteurianum*. Second, the level of substrate supplied was much higher than had been employed by other investigators. Although pyruvate, as well as many other substrates, had been supplied, usually it was at a level that supported good respiration. It turned out that very high levels, approximately 0.15 *M* were best, because the highly active phosphoroclastic system in the extracts decomposed the substrate so rapidly. Third, the cells were dried at a bath temperature of about 45 C in a rotary evaporator. They were inactivated if dried from the frozen state. Subsequently, we found that one of the components of nitrogenase is cold labile. Everyone previously had been solicitous of the enzymes and had kept them at low temperature to avoid inactivation, a practice which proved to be dead wrong.

Availability of consistently active cell-free preparations attracted an increasing number of workers to the field of biological N_2 fixation and studies ramified. Active cell-free preparations were made from several organisms, and Bulen *et al.* (8) suggested the helpful modification of using $Na_2S_2O_4$ as the electron donor for nitrogenase.

There had been interest, previously, in intermediates between N_2 and NH_3, but any nitrogen fixed was metabolized so rapidly by intact organisms that it had never been possible to recover any intermediates. It seemed that cell-free preparations should make it easier to demonstrate the occurrence of compounds at intermediate reduction levels. However, tests devised to recover intermediates such as diimide, hydrazine, or hydroxylamine from cell-free preparations vigorously fixing N_2, revealed no intermediates even with the sensitive tracer technique. No one else had any greater success, and it was concluded that intermediates must remain bound to the nitrogenase enzyme throughout the reduction process until they are finally released as NH_3.

It was obvious that detailed studies of nitrogenase required that the enzyme be purified. Mortenson and co-workers obtained evidence that there must be at least two components in nitrogenase. Mortenson (35) showed that nitrogenase consists of two proteins, a molybdenum iron protein and an iron protein. Neither of these has any catalytic activity by itself, but when the two are combined under proper circumstances, they will catalyze reduction of N_2. Each laboratory devised its own means for separating and purifying the protein constituents, but in several laboratories these proteins have been brought to homogeneity from aerobic, anaerobic, and facultative organisms, and the molybdenum iron protein from *Azotobacter vinelandii* has been crystallized (10).

For a time, biochemical studies of N_2 fixation centered on preparations from free-living organisms. However, Bond (7) continued an active program on the nonleguminous plants, Bergersen (5) obtained fixation of N_2 with breis from soybean root nodules, and Evans *et al.* (27) obtained fixation by bacteroids and extracts from the bacteroids. These studies not only established that symbiotic N_2 fixation in legumes was localized in the bacteroids rather than in plant tissue, but also showed that the role of hemoglobin in N_2 fixation must be indirect. After Kubo (28) identified hemoglobin in leguminous root nodules and suggested that it influenced respiration of the bacteria in the nodules, there had been much speculation that the hemoglobin functioned directly in the binding and reduction of N_2. Now we are essentially back at Kubo's position, but with the story much more fully developed (6). Dilworth (14) showed that two species of legumes infected with the same strain of the rhizobia produce different hemoglobins; it then was apparent that genetic information for the globin portion of hemoglobin is derived from the plant.

With methods available for obtaining pure enzyme components, biochemical and microbial geneticists were attracted to the field to attempt to control the production of either or both of the enzyme components by genetic manipulation. Genetic information has been transferred from *Klebsiella pneumoniae* to *Escherichia coli* (15, 43). Brill *et al.* (41) have been able to produce mutants deficient in either the molybdenum iron protein or the iron protein. A variety of mutants have supplied useful tools for further investigations of the mechanism of N_2 fixation. Many groups now are busily engaged in attempts to insert genetic information for N_2 fixation into nonleguminous plants, or to modify the proteins in such a way that N_2 fixation is not repressed by the ammonia they produce (20). The possibilities of extension of N_2-fixing capacity to other plants, of improvement in its efficiency, and of enhancing the mutual interaction of free-living bacteria and nonleguminous plants look bright. Genetics of N_2 fixation has attracted widespread research interest in recent years.

Nitrogenase has proved to be an unusually versatile enzyme. Obviously it binds N_2 and reduces it, but the first inkling that it could bind some other material came with the observation by Wilson and Umbreit (49) in 1937 that H_2 was bound at the same site. In 1954, Mozen (37) observed that nitrous oxide was reduced by *Azotobacter vinelandii* and by nodules from soybean plants. This constituted the first evidence that any substance other than N_2 was reduced by nitrogenase. We verified this in 1960 and extended it to cell-free preparations in 1965. In 1965, Schöllhorn, who was doing postdoctoral work in my laboratory, observed that azide and acetylene also were reduced by nitrogenase. In my correspondence with Dilworth at Christmas time, 1965, I discovered he independently had observed the reduction of acetylene. We had a lively correspondence for a few months and then published our observations independently. Schöllhorn was of the opinion that acetylene was a competitive inhibitor of N_2 fixation, whereas Dilworth's observations suggested that it was a noncompetitive inhibitor. Subsequent tests in our laboratory have shown that Dilworth was correct.

The observation of acetylene reduction led to the development of a simple technique for measuring N_2 fixation. A number of groups contributed to the method, and at the September 1973 International Biological Program conference in Edinburgh, I described the history of development of the acetylene reduction technique. It is necessary here only to say that the method has given tremendous impetus to field studies of N_2 fixation. The method has not always been applied wisely, but still it has been of real use in improving estimates of the quantity of N_2 fixed under natural conditions.

Hardy and Knight (22) in 1967 reported that cyanide was reduced by nitrogenase and Kelly *et al.* (26) in the same year year found that methylisocyanide also was reduced. Various analogs of a number of these compounds also are reduced by nitrogenase. The combination of the iron protein and the molybdenum iron protein plus ATP and a strong reductant will reduce protons to H_2 and will act as an ATPase. The enzymatic versatility of nitrogenase is evident.

These various substrates appear to be reduced from a common pool of electrons, and hence, they inhibit each other. Inhibitors of interest, in addition to those which can be reduced, include such compounds as nitric oxide and carbon monoxide, both of which are unusually strong inhibitors of

N_2 fixation. A study of the interaction of the various substrates and inhibitors suggests that the nitrogenase enzyme has multiple sites, or that the active site is modified by the presence of the various substrates. There are subtle differences among the substrates, despite the fact that reduction of each of them carries an absolute requirement for ATP and a strong reductant, and that both components of nitrogenase must be present to effect a reduction.

The early reports with active cell-free preparations indicated that ATP was an inhibitor of N_2 fixation (actually ADP and ATP in the absence of Mg^{2+} are inhibitors). When McNary and Burris (32) suggested that MgATP was required to support N_2 fixation, the idea met with considerable skepticism. However, when others checked the matter experimentally, they found in fact that there was an absolute requirement for MgATP, and that this extends to all of the reactions catalyzed by nitrogenase. It was not clear what the function of the ATP was other than the obvious assumption that it was supplying energy to support the reduction reaction.

Ideas on electron transport in N_2 fixation were given a boost when Mortenson *et al.* (36) reported that an electron carrier which they christened ferredoxin was present in clostridial extracts. They later showed that it could function in N_2 fixation, and others have found ferredoxins to be functional in other N_2-fixing organisms. The unusually low potential of this carrier fits it for the energy-demanding reduction of N_2. In *Azotobacter vinelandii,* a flavoprotein also can serve as an electron donor in N_2 fixation (4).

Despite the fact that it was recognized that ferredoxin was involved, schemes for electron transport in N_2 fixation depended more upon intuition than experimental data. This situation was improved immensely when it was found that the components of nitrogenase exhibit characteristic EPR[1] signals at liquid helium temperatures (21). The signals for the iron protein cluster around $g = 2.0$, whereas the signals for the molybdenum ion protein are around $g = 3.77$, 4.29, and 2.01. The positions of the signals vary somewhat with the source of the protein. The signal for the iron protein is altered in the presence of MgATP (38) and this observation, plus additional evidence (44) indicate that MgATP is specifically bound to the iron protein. The signals of the molybdenum iron protein disappear upon complete reduction (38) and during the physiological electron transfer of this component it shuttles between a partially and fully reduced state which can be monitored by EPR. There was some confusion in interpreting the EPR signals, but the Wisconsin (38) and the Sussex group (17) agreed that the physiologically important reaction is represented by the shuttle between the completely and partially reduced forms of the molybdenum iron protein. The Wisconsin group also observed that the binding of MgATP to the reduced iron protein is accompanied by a substantial lowering of its oxidation-reduction potential. This confers upon the iron protein the unique capacity to reduce the molybdenum iron protein. Although $Na_2S_2O_4$ is a potent reducing agent, it is incapable of completely reducing the molybdenum iron protein. EPR measurements have indicated that the binding of MgATP to the reduced iron protein confers upon it the ability to reduce the molybdenum iron protein, an ability which is the key to the interaction of the iron protein and the molybdenum iron protein of nitrogenase. Molybdenum iron protein apparently binds substrate, accepts electrons from the iron protein, and effects the reduction of the substrate with the liberation of the reduced products. These data are the basis for the concept of the mechanism of N_2 fixation shown in Figure 1.

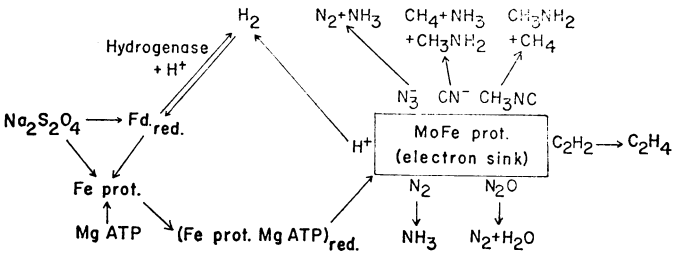

FIG. 1. Currently proposed mechanism for biological N_2 fixation. Reduced ferredoxin or $Na_2S_2O_4$ reduces the iron protein of nitrogenase. When the reduced iron protein binds MgATP, its potential is lowered to about −490 mv, and it acquires the unique capacity to reduce the molybdenum iron protein of nitrogenase. The reduced molybdenum iron protein serves as an electron sink for the reduction of the various substrates of nitrogenase, and the molybdenum iron protein becomes reoxidized in the process. The four sides of the electron sink rectangle represent enzyme sites modified to give each special characteristics, *e.g.* N_2 and N_2O compete for a common site, and N_3^-, CN^-, and CH_3NC compete for a common site. H_2 evolution via hydrogenase does not require ATP but is CO-sensitive, whereas H_2 evolution via nitrogenase requires ATP but is insensitive to CO.

A number of problems remain for future resolution of the mechanism of N_2 fixation. For example, we have very sketchy evidence to support the binding of substrates to the molybdenum iron protein. The rate-limiting reaction must be defined. The mode of action of carbon monoxide must be determined, because it inhibits all nitrogenase reactions except for the production of H_2. The point at which ATP is hydrolyzed has not been established, and data supporting formation of a persistent complex between the iron protein and the molybdenum iron protein still are controversial.

In the last decade or so, the study of biological N_2 fixation first became respectable and then became highly popular. Except for work at the Fixed Nitrogen Laboratory, research on biological N_2 fixation had been performed primarily at universities and experiment stations rather than at laboratories organized to investigate N_2 fixation. The Kettering Laboratories designated N_2 fixation together with photosynthesis as major research targets. The du Pont laboratories are unique among industrial firms in maintaining a continuing program of basic studies on N_2 fixation. Commonwealth Scientific and Industrial Research Organization laboratories in Canberra and the Agricultural Research Council Unit of Nitrogen Fixation associated with the University of Sussex represent creation and continuing support of major research units with sizeable senior staffs. All of these units have been highly productive and have fully justified decisions which backed continuing and substantial support of work on N_2 fixation.

Another index of interest in N_2 fixation is the number of books published on the subject. There was a long gap between Wilson's 1940 monograph and Stewart's book in 1966 (42). Then the publishing gap narrowed with books by Mishustin and Shil'nikova (34) in 1968 (English translation, 1971) and Postgate (39) in 1971. At the moment, books edited by Hardy and by Quispel together with a volume derived from the International Biological Program at Edinburgh in 1973 are promised for early publication.

A number of things have contributed to the tremendous expansion of interest in biological N_2 fixation in recent years, among them the general expansion in biological sciences, the interest in the role of N_2 fixation in eutrophication of lakes (18), the advent of the acetylene reduction technique which makes it relatively easy to study fixation in the field, the concern with toxicity of nitrates and nitrites arising from fertilizers, and the observation that progress is being made in re-

[1] Abbreviation: EPR: electron paramagnetic resonance.

search on N_2 fixation. A concern with increased food productivity, and particularly the rapid rise in popularity of soybeans as a crop, have sparked interest in the study of the symbiotic N_2 fixation system. The fact that nonsymbiotic bacteria associated with the roots of nonleguminous plants can be responsible for substantial N_2 fixation also has triggered expanded research in this area. Finally, the energy crisis has brought people to the realization that dependence upon N_2 fixation by the Haber process carries with it a demand for a tremendous energy expenditure, an energy demand which has dictated a substantial rise in the cost of nitrogenous fertilizers. It is recognized that as an alternative to the Haber process, the agricultural economy can depend more extensively on biological N_2 fixation, a process which derives its energy from the sun.

Research on biological N_2 fixation grew modestly for a long period. As the importance of the process has become more widely recognized, there has been a surge of interest in intensifying research on N_2 fixation. The research has been productive in clarifying old problems and opening new horizons.

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